Defining Your Product Profile and Maintaining Control Over It, Part 3

Product-Related Impurities

Renee Boerner and Kathleen Clouse

Biological product characterization involves determining product physicochemical properties, biological activities, and immunochemical properties, as well as assessing product purity and impurities. Product-related variants arise because of the heterogeneity associated with biological systems and can also occur during manufacturing, handling, and storage. Although ICH Q6B defines product-related substances and impurities and discusses the setting of specifications, it does not describe how to approach individual situations and variants. As a result, numerous questions still exist.

A Strategy Forum

On 20 July 2004, the Well-Characterized Biotechnology Product (WCBP) Chemistry and Manufacturing Controls (CMC) Strategy Forum workshop entitled "Product-Related Impurities" was held in Bethesda, Maryland, to discuss the general strategies for maintaining control over product-related impurities. The panelists in this session were Renee Boerner (Diosynth-Biotechnology), Kathleen Clouse (CDER, FDA), Dieter Schmalzing (Genentech), and Laura Bass (Pfizer) (see the "Proceedings" box for more information.)

Two separate workshops were held as a part of the Strategy Forum. The first workshop was designed to introduce and define product-related impurities and product-related substances (collectively known as product-related variants), to hear two case studies of how such variants were handled, and to discuss general strategies for setting specifications on product-related variants. This article summarizes the discussion that occurred and describes some general approaches commonly used in the industry.

Discussion began with the following questions:

- How are product-related impurities and product-related substances defined?
- How do you distinguish between critical and noncritical product-related impurities?
- How are specifications set for product-related impurities/substances?

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Workshop participants agreed on and outlined the following steps toward addressing those questions: characterizing variants, classifying each variant as a product-related substance or a product-related impurity, assessing whether the variant must be controlled, and determining specifications for those variants that need control.

Characterization

Before a product-related variant can be considered an impurity, that variant must be characterized. The consensus was that characterization should begin as early as possible in the product development cycle. Many agreed that variants present at significant amounts (defined by some as levels of 1% or greater) should be targeted for characterization. But considerations

Product Focus: Recombinant proteins

Process Focus: Process Development (product characterization)

Who Should Read: Manufacturing and process development, project managers, and analytical personnel

Keywords: Glycosylation, microbial and animal cells, aggregation, deamidation, protein varients; and product-related impurities, substances, and variants

Level: Basic

The sixth Well-Characterized Biotechnology Pharmaceutical (WCBP) Chemistry, Manufacturing, and Controls (CMC) Strategy Forum was held on 19–20 July 2004 at the Lister Hill Auditorium on the NIH Campus in Bethesda, Maryland. The event was sponsored by the California Separation Science Society (CaSSS; www.casss.org) as part of an ongoing series of discussions between industry and regulatory participants exploring current practices in analytical and bioprocess technologies for development and communication of consensus concepts. The topic of this forum was "Defining Your Product Profile and Maintaining Control Over It."

The purpose of this two-day forum was to survey which methods are most useful in identifying and measuring process-related and product-related impurities — and identify strategies and specifications to ensure a consistent product profile. The first day concentrated on process-related impurities (Parts 1 and 2 of this article); on day two the focus shifted to product-related impurities (Parts 3 and 4).

Participants on the second day discussed general strategies for maintaining control over product-related impurities. The moderators were Renee Boerner (Diosynth-Biotechnology) and Kathleen Clouse (CDER, FDA); they were joined on the panel by Dieter Schmalzing (Genentech) and Laura Bass (Pfizer).

The members of the permanent CMC advisory committee are Siddharth Advant (Diosynth Biotechnology), John Dougherty (Eli Lilly and Company), Rohin Mhatre (Biogen Idec Inc.), Anthony Mire-Sluis (Amgen, Inc.), Wassim Nashabeh (Genentech, Inc.), Nadine Ritter (Biologics Consulting Group, LLC), Mark Schenerman (MedImmune, Inc.), Heather Simmerman (Amgen, Inc.), and Keith Webber (CDER, FDA).

163 people attended the two-day conference, representing industry companies, consultant companies, and the FDA.

should also be given regarding the actual concentration of the variant and whether chronic dosing is anticipated. This is often subject to evaluation case-by-case. Characterization entails identifying the variant, the specific site(s) of modification or alteration, the amount of variant present, and the way in which it is formed. This process usually requires separating the variant from the desired product. Another approach is to look specifically for expected modifications, such as by using mass spectroscopy to look for +16 Da species that could suggest the presence of methionine oxidation variants.

A number of analytical methodologies can be used for characterization, depending on the nature of the variant. Often times, analytical methods needed to characterize variants are unavailable when preclinical and clinical materials are manufactured, so it is important that sufficient retains of those materials be adequately stored to enable analysis of variants when appropriate assays are identified.

Product-Related Variants and Relevant Analytical Methodologies: One important point is that, in some cases, sample handling during analytical analysis can actually form or mask variants. Care must therefore be exercised to ensure that analytical procedures do not introduce or destroy variants.

Adducts (chemical addition products) can form as a result of sample processing. For example, cysteine or glutathione adducts can form when cysteine or glutathione is used to keep proteins in a reduced state or to aid in refolding. In general, adducts can be readily identified using mass spectrometry.

Aggregation and oligomerization are common variants, often identified by size-exclusion chromatography. (Aggregation is the topic of Part 4 of this series, scheduled for *BioProcess International*'s November issue.)

Examples of **amino acid substitutions** have been identified, such as the incorporation of norleucine for methionine (**1–3**) or incorporation of norvaline for leucine (**4**) under growth conditions in which certain amino acids were limited.

Carbamylation is seen in some proteins for which processing involves high concentrations of urea under conditions that promote cyanate production (**5, 6**). Cyanate production increases at high pH and elevated temperatures. Once formed, isocyanate can attack the free amino group of the N-terminus as well as the free amino groups of lysines. Trypsin and lys-C are unable to cleave modified lysine residues, so carbamylation of specific residues can be identified by peptide mapping experiments.

Protein fragmentation is also common, resulting from N-terminal and C-terminal truncations or internal cleavage. Methods that examine the integrity of the termini (such as N-terminal sequencing or peptide mapping in combination with mass spectrometry analysis) can usually identify the cleavage sites. In some cases, unless the protein is analyzed in its reduced form, internal cleavages may not be obvious if the protein is still held together by disulfide bonds (**7**). Intracellular expressed proteins are often susceptible to cleavage from proteases upon cell lysis. C-terminal lysine removal has been observed in antibody heavy chains (**8, 9**).

Deamidation of asparagine residues is facilitated by high pH and occurs commonly in the presence of asparagine-glycine sequences. Asparagine deamidation occurs through formation of a five-member succinimide ring. Hydrolysis typically results in formation of a 3:1 ratio of isoaspartate:aspartate residues (**10**). Succinimide variants have been detected in proteins, including a human growth hormone in which a succinimide was detected at Asp-130 (**11**). Deamidation of glutamine residues in proteins is possible, but not common, given that the rate of formation is significantly slow.

Deamidation is commonly detected by method separations based on charge. In monoclonal antibodies it can be detected using IEX-HPLC and isoelectric focusing (**12**).

Spelling Them Out

Here are some abbreviations used in this article.

CE: capillary electrophoresis

DSC: differential scanning calorimetry

HIC-HPLC: hydrophic-interaction HPLC

HPLC: high-performance liquid chromatography

IEX-HPLC: ion-exchange HPLC

MALDI-TOF: matrix assisted laser-desorption ionization–time of flight

PEG: polyethylene glycol

RP-HPLC: reversed-phase HPLC

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

Isoaspartate residues produced as a function of deamidation or isomerization can be detected using the Isoquant assay kit (Promega, www.promega.com).

Correct disulfide formation is usually required for appropriate protein function and stability. Incomplete disulfide formation or scrambled disulfides can result in a protein that is misfolded or not as thermodynamically stable. Although disulfide mapping of proteins is recommended to confirm or identify disulfide bonds, many other techniques can be also be used. Differences in disulfide bonding patterns and structural integrity dictated by disulfide bond formation can be analyzed by methods including but not limited to nonreducing SDS-PAGE, RP-HPLC, IEX-HPLC, HIC-HPLC, and DSC. Trisulfides also can form where disulfide bonds normally occur. Trisulfides contain a sulfur atom covalently bound by two cysteine residues and can be shown by mass spectrometry as detected by an increase in mass of 32 Da species (**13, 14**).

N-linked and O-linked glycosylation are posttranslational modifications observed in eukaryotic expression systems. O-linked glycosylation can occur on serine and threonine sites, and N-linked glycosylation targets asparagine residues, which reside in the consensus sequence asn-X-ser/thr/cys (where X cannot be proline).

A number of N-terminal modifications can occur, including acetylation and pyroglutamate formation. Acetylation occurs most commonly on serine and alanine residues (**15**). In one case, the mutation of the N-terminus to a serine was introduced as a way of inducing N-terminal acetylation and preventing N-terminal cleavages in the unmodified protein. Acetylation can be detected as an increase in mass of 42 Da. Pyroglutamate formation is commonly observed in antibodies that contain glutamate or glutamine residues at their N-termini. A pyroglutamate is formed when the amino group and the carboxylic group (glutamate) or carboxamide group (glutamine) condense, forming a five-membered ring. Pyroglutamate formation can be identified as a loss of 17 or 18 Da when formed from glutamate and glutamine residues, respectively.

Oxidation is most commonly observed on methionine residues, giving rise to a +16 Da sulfoxide species. Forced degradation studies in which hydrogen peroxide or t-butyl hydroperoxide is incubated with proteins are often used to identify sites most susceptible to oxidation and are a common route for investigating the effect of oxidation on activity (**16, 17**).

PEGylation is frequently used to enhance the pharmacokinetics of protein therapeutics (**18**). It is frequently targeted at free amino groups in the N-terminus (**19**) or surface exposed lysine residues (**20**). Analytical methods for PEGylated proteins can be quite challenging, especially when multiple PEGylated isomers are present. CE and MALDI-TOF mass spectrometry have previously been used to characterize the number of PEGs attached to a single molecule.

Phosphorylation is a posttranslational modification that can occur on serine, threonine, and tyrosine residues. It can be detected by mass spectrometry as an increase of 80 Da or by Western analysis using specific antibodies capable of detecting phosphorylated residues.

Classification

Key discussion questions addressing ways to classify variants are

- How do you define product-related impurities and product-related substances?
- Do variants have comparable properties or activities?
- What does *comparable* mean?

To assess whether a product-related variant should be considered an impurity, data are collected to determine whether that variant has comparable activity, efficacy, and safety. In concept, the activity of the variant can be measured directly if variant-enriched fractions can be made or if the variant can be isolated in a relatively pure form. The variant-enriched fraction and the desired molecule (which ideally has gone through similar handling procedures as the variant) are then assessed to see whether the variant has comparable activity or whether its activity falls outside of the potency specification.

However, determination of comparable activity hinges on the accuracy of the potency assay and its relevance to the physiological activity of the molecule. The wrong conclusion could be made if an assay isn't sensitive enough to detect subtle differences or if it is not relevant to the mechanism of action for the product. Some situations may require multiple assays. For example, a binding assay may need to be used in conjunction with a cell-based potency assay. The binding assay may actually be more sensitive to product changes, whereas the cell-based assay may be more physiologically relevant. In general, variants with altered activity would be characterized as product-related impurities. Variants with comparable activity would then be further evaluated for efficacy and safety to determine whether they are impurities.

Unfortunately, because it is neither feasible nor practical to perform these studies on individual variants, it can be difficult to get a direct readout of the impact of product variants on safety and efficacy. Indirect approaches may be needed to determine whether a variant is likely to have an effect on safety. One such approach is to assess whether the variant is expected or unexpected and/or unusual. In addition, consideration should be given to the in vivo occurrence of the

variant: whether it is present at levels less than or equal to those observed in material used previously for preclinical and clinical studies and the current regulatory climate. There is at present a heightened concern regarding aggregates, so the regulatory climate suggests taking a conservative approach for them as well as other variants known to affect product safety.

The audience was specifically asked to provide examples of variants known to negatively affect product activity, efficacy, or safety, but only a small number of cases were mentioned at the workshop (Table 1). Many more cases have been discussed in the literature. As more and more data become available, it may be possible to take a more proactive approach in determining whether a variant should be classified as an impurity. For this reason, participants recommended that additional data be collected and compiled into a single database to be shared by industry and regulatory groups.

Table 1: Variants known to negatively affect product activity, efficacy, or safety as identified in examples offered by the forum participants

Heterogeneity	Affects Safety	Affects Activity	Affects Efficacy
Adducts		Free SH group in MAb binds to cysteine and is inactivated	
Aggregation or multimers	May lead to immunogenicity		
Disulfide formation or scrambling		Disulfide scrambling reduced potency	
Glycosylation	May lead to immunogenicity: sialylation affected PK in an enzyme product; desialylation reduced bioavailability of Factor VIII; when produced in milk from transgenic sheep, high amounts of N-glycolated (neurominic acid) were observed, which is very immunogenic	May be necessary for activity: Galactosylation affects activity of antibody	May influence efficacy through sialylation; efficacy is dependent on glycoforms
Misfolded protein		Misfolded proteins have no biological activity	
Oxidation		Oxidation of methionine in a monoclonal antibody affected the binding assay	
PEGylation	Affect on PK due to PEG size, PEG differences, PEG distributions	May reduce activity	May increase efficacy through increased bioavailability

Control

Some key questions addressed the degree to which a variant needs to be controlled:

- How do you distinguish between critical and noncritical product-related variants?
- What is meant by *critical*?
- Does the variant reflect manufacturing consistency and/or affect safety and efficacy?

If a variant has an impact on safety, efficacy, or bioavailability, it is critical. Other critical variants are those indicative of manufacturing consistency and those that change on stability or during handling. Variants of heightened regulatory concern, such as aggregates, might also be considered critical because of the impact that a specific type of variant may have had in another protein product. All critical variants should be controlled and reduced as much as possible. Control needs to be established so that the material going into patients is as pure (in terms of total impurities and individual variants) as the material used in previous clinical studies. When asked whether a product should intentionally be made less pure for early clinical trials, the consensus was that using a less pure product may run the risk of increasing adverse events. Therefore, locking down a good process as early as possible and controlling the process to maintain consistency becomes the preferred approach to minimize potential adverse events.

Specifications

Some of the key questions regarding setting specifications were

- What is the strategy for setting specifications for product-related variants?
- How many variants do you set specifications for?
- Which variants do you set specifications for?
- When do you set specifications?

The participants agreed that no hard and fast rules exist for determining required purity levels or an acceptable threshold for impurities. Similarly, no rules identify the number of variants for which specifications must be set. Many sponsors start by reporting values of individual variants during clinical studies and are careful not to assign too many specifications or those that are too narrow at that early stage. However, many workshop attendees felt that preliminary specifications must be set. In general, a logical path is to set wide specifications early on and then narrow them as experience is gained. In some cases, though, it may be acceptable to proceed without a specification if the impurity is not present at concentrations ample enough to trigger an immune response, if it is observed early in clinical trials and is within the range of clinical experience, and if no negative impact is observed.

Reaching a Conclusion

The November issue of *BioProcess International* will conclude this series of reports from the 19–20 July 2004 WCBP CMC Strategy Forum with Part 4 addressing aggregation. The authors of that article are Kurt Brorson (Office of Biotechnology Products, CDER, FDA) and Joseph Phillips (senior director, analytical sciences, Amgen, Inc).

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